# DISTINCT PHENOTYPIC CHANGES BY PACAP AND VIP IN LIPOPOLYSACCHARIDE-STIMULATED BV2 MICROGLIAL CELLS

Jocelyn Karunia[1], Aram Niaz[1], Mawj Mandwie[1], Ghaith Al-Badri[1], **Alessandro Castorina**[1,2]

[1] Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, University of Technology Sydney, NSW, Australia

[2] Discipline of Anatomy & Histology, School of Medical Science, The University of Sydney, NSW, Australia

## Background

Microglia are resident glial cells of the brain. These cells regulate brain development, maintain homeostasis of neuronal networks, and promote injury repair [1]. Due to their phagocytic activity, microglia serve as brain macrophages, but are distinct from other tissue macrophages owing to their unique phenotype and tight regulation by the central nervous system (CNS) microenvironment [2]. However, there is evidence suggesting that aberrantly activated microglial cells play a key role in the progressive neuronal loss seen in many neurodegenerative diseases [3]. Pituitary adenylate cyclase-activating polypeptide (PACAP) and the homologue vasoactive intestinal peptide (VIP) are two neuropeptides, both which elicit robust immunosuppressive functions within the CNS [4, 5]. Nonetheless, a number of studies have suggested that PACAP or VIP may elicit immune suppression through differing mechanisms on microglial cells [6].

## Aims

In this study, using lipopolysaccharide (LPS) to induce BV2 microglial cell polarisation, we aimed at testing whether and how administration of either PACAP or VIP could differentially affect microglial pro-inflammatory profile, polarisation state and morphological appearance to elicit immunosuppressive effects.

## Methods

### BV2 microglial cell cultures

Murine BV2 microglial cells were cultured in DMEM/F12 and supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and allowed to grow at 37°C in a humidified atmosphere with 5% $CO_2$. Cells were used between passage 25-35.

### Real-time qPCR

Total RNA was reverse transcribed as per Bioline Tetro cDNA synthesis kit protocol and transcripts were amplified using SYBR green technology using the BioRad CFX96 Touch Real-Time PCR Detection System. Primers were optimised for qPCR. S18 ribosomal protein subunit was used as housekeeping gene. $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes [7].

### Western blot analysis

Sample proteins (30µg) were diluted in Laemmli (BIO-RAD) and β-mercaptoethanol (SIGMA-ALDRICH) mixture, heated at 70°C for 10 min and then separated on a BIORAD 4-20% gradient criterion mini gels (BIO-RAD).

### Immunocytochemistry

Cells cultured on glass coverslips were fixed in 4% PFA, permeabilised with 0.2% Triton X-100, blocked with 0.1% BSA in PBS, then probed with the appropriated primary antibodies.

### Griess reagent assay

Levels of nitric oxide (NO) in supernatants were quantified by the Griess reaction. Cells ($5\times10^5$) were treated with LPS in the presence or not of PACAP and VIP for 12h. Media was then replaced with freshly prepared one and incubated for a further 24hr. 100µl Griess reagent (Sigma-Aldrich, St. Louis, MO) was added to an equal volume of conditioned media in a 96-well plate. Absorbance was read using at an absorbance of 540nm using a Tecan infinite M1000 Pro ELISA reader.

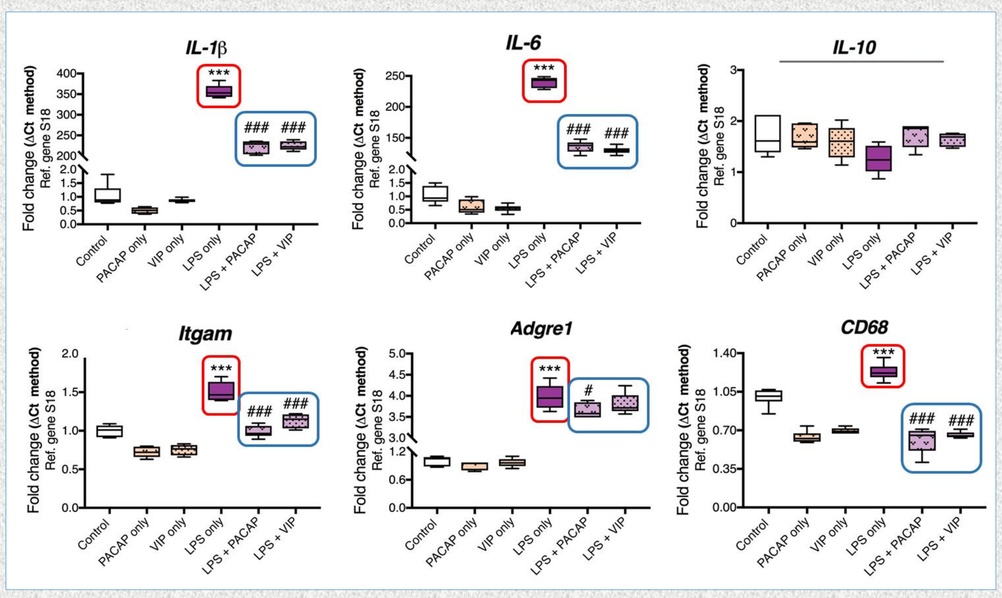
### Morphological analyses

$5\times10^5$cells were exposed to treatments for 12h as follows: Control, LPS only, LPS+PACAP and LPS+VIP. Images were then captured using the Nikon Eclipse TS2 inverted microscope and subjected to morphological assortment and analyses. Specific criteria were decided on and blinded individuals assigned randomised images to analyse (see **Table 1** for phenotypic criteria). Cells were grouped in their classifications; a) small; b) medium; c) large and a) rounded; b) bipolar/spindle; c) multipolar. Analyses of embossed microscope images was performed using ImageJ 1.51 (NIH) software.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism software. Comparisons were computed using one-way ANOVA followed by Tukey or Sidak's *post-hoc* tests, as appropriate. P values <0.05 were accepted as statistically significant.

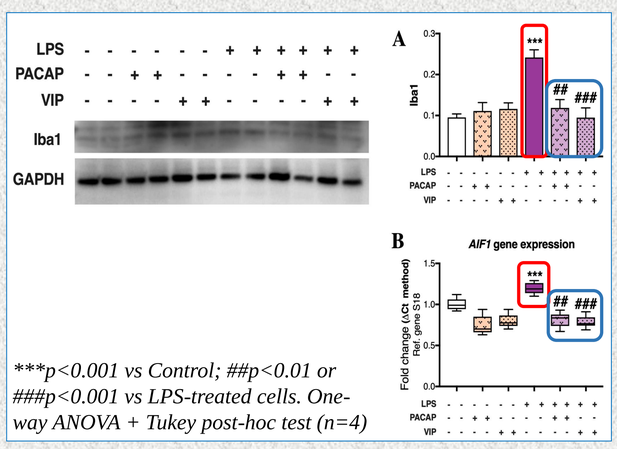
## Results



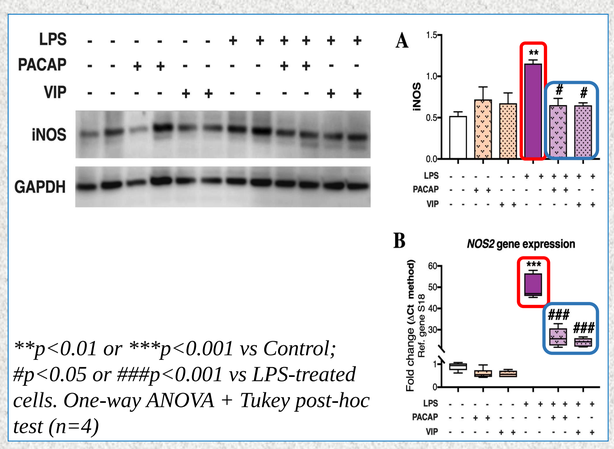

**(1) PACAP & VIP reduce pro-inflammatory cytokine mRNAs in LPS-treated BV2 cells.**

BV2 cells exposure to 1µg/ml LPS for 12hrs significantly increased the expression levels of several pro-inflammatory cytokines (circled in red). Treatment with 100nM PACAP or VIP reduced cytokines' mRNA levels back to normal in most cases (circled in blue). IL-10 levels did not seem to be affected by LPS or peptide treatments.

****p<0.001 vs Control; #p<0.05 or ###p<0.001 vs LPS-treated cells, as determined by one-way ANOVA followed by Tukey post-hoc test (n= 4-6)*



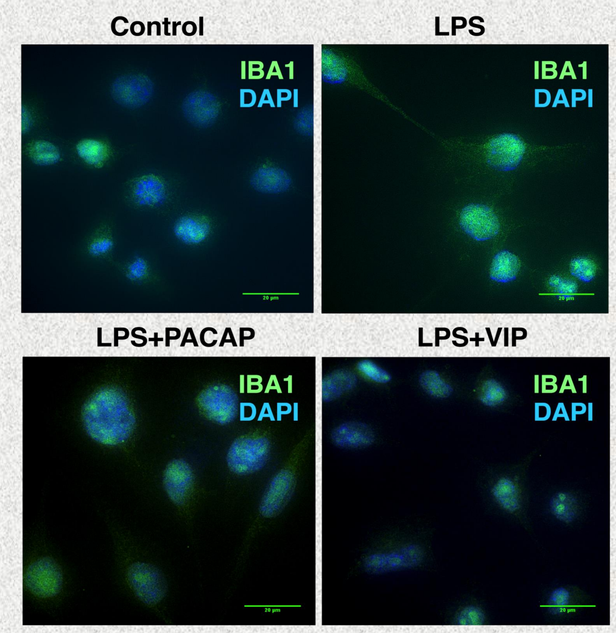

****p<0.001 vs Control; ##p<0.01 or ###p<0.001 vs LPS-treated cells. One-way ANOVA + Tukey post-hoc test (n=4)*

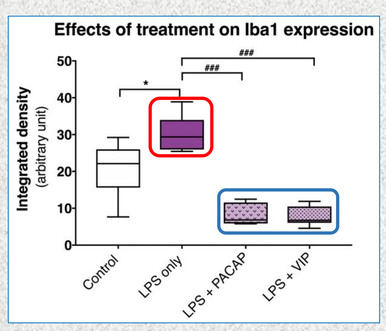


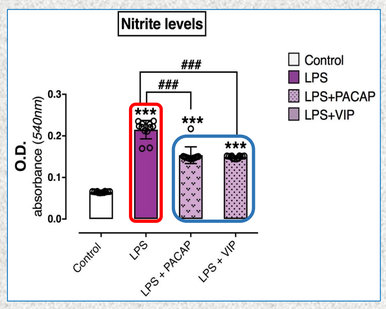

***p<0.01 or ***p<0.001 vs Control; #p<0.05 or ###p<0.001 vs LPS-treated cells. One-way ANOVA + Tukey post-hoc test (n=4)*

**(2) PACAP & VIP reduce Iba1 and iNOS levels in LPS-treated BV2 cells.**

LPS-activated BV2 cells exhibit increased levels of M1 polarization markers, both of which are significantly reduced upon treatment with PACAP or VIP.



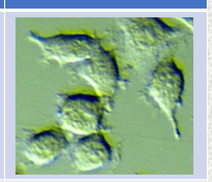
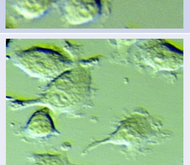
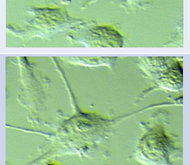

**(3) PACAP & VIP decrease LPS-induced Iba1-like immunoreactivity (IR) and nitrites production in BV2 microglia.**

Iba1-like IR was increased by treatment with 1µg/ml LPS at 12h *(*p<0.05)*. Similarly, nitrite levels were also augmented by LPS (****p<0.001*). PACAP or VIP rescued both Iba-like IR and nitrite levels in treated cells (###p<0.001).

*IHC-related statistics:*p<0.05 vs Control; ###p<0.001 vs LPS-treated cells as determined by one-way ANOVA followed by Sidak post-hoc test (n=6)*

*NO assay-related statistics: ***p<0.01 vs Control; ### p<0.001 vs LPS-treated cells as determined by one-way ANOVA followed by Tukey post-hoc test (n=18)*

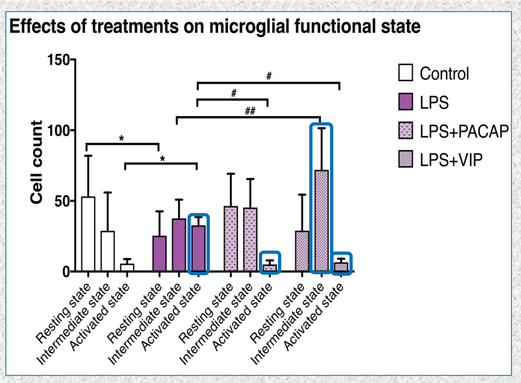
**TABLE 1**

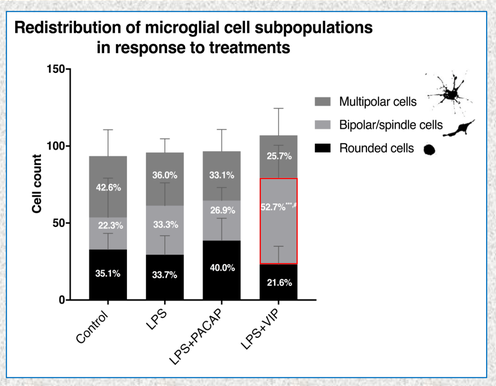
| Phenotype | Descriptions | Visual Example |
|---|---|---|
| Small cells/resting | Round, oval-shaped cells with small soma and thin, long processes |  |
| Medium cells/intermediate | In between the two phenotypes |  |
| Large cells/activated | Multipolar or spindle-shaped, flattened cells with hypertrophied soma and retracted, thick processes |  |

**(4) VIP but not PACAP increase the number of intermediate/spindle shaped cells in LPS-treated BV2 microglia.**

*Functional state statistics:*p<0.05 vs Control; #p<0.05, ##p<0.01 vs LPS-treated cells as determined by 2-way repeated measures ANOVA followed by Tukey post-hoc test (n=40)*

*Subpopulation-related statistics: ***p<0.001 vs Control; #p<0.05 vs LPS-treated cells as determined by 2-way RM ANOVA followed by Tukey post-hoc test (n=40)*




## References

1. Hickman S et al., (2018), Nat Neurosci. 21(10):1359-1369.
2. Kierdorf K al., (2019), Nat Rev Neurosci. 2019 Sep;20(9):547-562.
3. Colonna M & Butovsky O (2014), Annu Rev Immunol. 26;35:441-468.
4. Waschek JA (2013), Br J Pharmacol. 169(3):512-23.
5. Ganea D et al., (2003), Cell Mol Biol (Noisy-le-grand). 49(2):127-42.
6. Abad C & Tan YV (2018), J Mol Neurosci. 66(1):102-113.
7. Schmittgen & Livak (2008), Nat Protoc. 3(6):1101-8.

## Take home message

Taken together, these results demonstrate that both PACAP and VIP possess robust anti-inflammatory activities in experimentally activated BV2 microglial cells. However, from a morphological perspective, it appears that the two peptides trigger distinct shifts of certain cell subpopulations towards specific phenotypes. Further studies are warranted to unveil which biological functions are likely to be affected by these changes in cellular architecture.